UNIVERSITI PUTRA MALAYSIA

THE AMELIORATIVE EFFECTS OF NIGELLA SATIVA ON THE OXIDATIVE STATUS AND THE PATHOLOGY OF STREPTOCOCCUS AGALACTIAE INFECTION IN RED HYBRID TILAPIA (OREOCHROMIS SP.)

NURAKMALIAH BINTI RAHAMAT@RAHMAT

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A project paper submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "The Ameliorative Effects of *Nigella sativa* on the Oxidative Status and the Pathology of *Streptococcus agalactiae* Infection in Red Hybrid Tilapia", by Nurakmaliah binti Rahamat@Rahmat and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999-

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DEDICATIONS

The final year project thesis is dedicated to my beloved family, all the lecturers, and friends that were involved either directly or indirectly in this project.

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LIST OF ABBREVIATION

N. sativa	*Nigella sativa*
S. agalactiae	*Streptococcus agalactiae*
PCR	Polymerase chain reaction
BHI	Brain heart infusion
CFU/mL	Colony forming unit per milliter
MDA	Malondialdehyde
SOD	Superoxidase dismutase
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic acid
L	Liter
mL	Milliliter
µL	Microliter
>	More than
<	Less than

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada VPD 4999 – Projek ilmiah tahun akhir

KESAN PEMBAIKAN PENGGUNAAN *NIGELLA SATIVA* TERHADAP STATUS OKSIDATIF DAN PATOLOGI DARIPADA JANGKITAN *STREPTOCOCCUS AGALACTIAE* DALAM TILAPIA MERAH (*OREOCHROMIS* SP.)

Oleh

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2015

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Jintan hitam adalah tumbuhan semulajadi yang boleh digunakan sebagai antioksidan selain daripada kegunaan propilaktik. Streptococcosis telah menjadi satu cabaran global dalam industri akuakultur di seluruh dunia. Kajian ini dilakukan bertujuan untuk menyiasat pengaruh jintan hitam terhadap status oksidatif dan patologi daripada jangkitan *Streptococcus agalactiae* dalam tilapia merah. Seratus dua puluh ikan anggaran berat 50 ke 150 g telah dipilih secara rawak dan dibahagikan kepada empat kumpulan, A, B, C dan D iaitu 20 ekor untuk setiap kumpulan dengan gandaan. Kumpulan A adalah kawalan negatif dan kumpulan B adalah kawalan positif di mana

kedua-dua kumpulan diberi makan makanan komersial sahaja. Kumpulan C dan D adalah kumpulan rawatan yang diberi makan dengan makanan ikan komersial dan gabungan 8 % dan 15 % jintan hitam setiap kumpulan selama 14 hari. Pada akhir kajian, semua ikan dalam kumpulan B, C dan D diinfeksi dengan *S. agalactiae*, 10^7 CFU/mL pada kadar 100 µL melalui intraperitoneum. Sampel darah dan tisu diambil pada hari ke-7, 14, 17, dan 18 selepas diberi makanan. Plasma digunakan untuk malondialdehid (MDA) manakala hemolysit digunakan analisa superoksida dismutase (SOD). Analisis statistik daripada nilai MDA dan SOD menunjukkan bahawa terdapat perbezaan yang signifikan antara kumpulan kawalan dan rawatan. Penemuan histopatologi pada 18 jam selepas jangkitan (hari ke-18 diberikan makanan) memperlihatkan keputusan signifikan antara kumpulan. Berdasarkan keputusan kajian ini, gabungan jintan hitam dalam makanan ikan dapat memberikan kesan yang baik terhadap tekanan dan meminimakan kadar kematian ikan yang disebabkan oleh streptococcosis.

Kata Kunci: Jintan hitam, Tilapia merah, *Streptococcus agalactiae*, biomarker tekanan, histopathologi.

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial for the course VPD 4999 – Final year project.

THE AMELIORATIVE EFFECTS OF *NIGELLA SATIVA* ON THE OXIDATIVE STATUS AND THE PATHOLOGY OF *STREPTOCOCCUS AGALACTIAE* INFECTION IN RED HYBRID TILAPIA (*OREOCHROMIS* SP.)

By

Nurakmaliah binti Rahamat@Rahmat

2015

Supervisor: Assoc. Prof Dr. Md Sabri Mohd Yusoff

Nigella sativa is a natural plant that could be used as antioxidant in addition to its prophylactic usage. Streptococcosis on the hand has become a global challenge to aquaculture industry all over the world. This study aimed at investigating the influence of *Nigella sativa* on oxidative stress and the pathology of *Streptococcus agalactiae* infection in Red hybrid tilapia. Hundred twenty fish weighing 50 to 150 g were randomly divided into four groups, A, B, C and D of 20 animals each with replicates. Group A was negative control while B was positive control in which both were fed with 100 % commercial diet. Group C and D were treatment groups fed with commercial diet incorporated with 8 % and 15 % *N. sativa* for 14 days, respectively. At the end of

experiment, all the fish in group B, C and D were challenged with *S. agalactiae*, 10^7 CFU/mL at the rate of 100 µL, intraperitoneally. Blood and tissue sampling were taken at day 7, 14, 17, and 18 post-feeding. Plasma was harvested and used for malondialdehyde (MDA) while haemolysates were used for superoxidase dismutase (SOD) analysis as stress biomarkers. Statistical analysis from the value of MDA and SOD indicated that there were significant different between controls and treatments' group. Histopathology examination at 18 hours post-infection (day 18 post-feeding) revealed significant findings between the groups. The incorporation of *N. sativa* in fish feed could ameliorate the stress and minimize the mortalities caused by streptococcosis.

Keywords: *Nigella sativa*, Red hybrid tilapia, *Streptococcus agalactiae*, stress biomarkers, histopathology.

1.0 INTRODUCTION

1.1 Study background

The tilapias (*Oreochromis* sp.) are freshwater fish that belong to the family Cichlidae, and they are exclusively associated with Africa and Middle East (Trewaves, 1983). According to FAO (2004), tilapias are among the most cultured fish worldwide. About 80 % of the global farmed tilapia production in 2002 showed Asia as the largest world producer. Among the Asian countries, Malaysia is listed among the top ten producers of farmed tilapia (El-Sayed, 2006). Recent survey has shown that an increase trend of cage fish culture activities have been operated in 6000 fish cages growing mostly red hybrid tilapia. Red hybrid tilapia is the main species cultured in Malaysian freshwater cage-culture system with production of 5664.42 tonnes valued at RM 36.38 million in 2010 (Department of Fisheries, 2010).

Initially, tilapias were considered to be more resistant to bacterial, parasitic, fungal, and viral diseases compared to other species of cultured fish. In more recent times, however, tilapias have been found to be susceptible to both bacterial and parasitic diseases. Streptococcosis is a disease that develops following the infection by *Streptococcus* sp. According to Yang and Li (2009), streptococcosis has been recognized as one of the most serious bacterial diseases in tilapia culture and usually causes high mortality and lasts a long period of time. *Streptococcus iniae* and *Streptococcus agalactiae* are the major bacterial species that affect the production of tilapias in the world (Evan et al., 2006). Ferguson et al. (1994) reported a mortality of

100 % in an experimental *S. agalactiae* infection as against low mortality in other environmental bacterial infections.

Streptococcus agalactiae was frequently isolated from cases of massive mortality of cage –cultured red hybrid tilapia in Kenyir and Pergau lakes in 2002 to 2003 (Siti Zahrah et al., 2004; 2005). This incidence was found to be related with seasonal changes and poor water quality, which affected the physiological conditions of the fish. Similar incidents also occur in Sungai Pahang cage-cultured fishes with mortality of 70 %. Erythromycin, florfenicol, and amoxicillin have been commonly used for the treatment of the streptococcosis (Treves-Brown 200; Darwish & Hobbs 2005; Yanong et al. 2005). In Malaysia, farmers tend to use erythromycin and oxytetracycline to treat streptococcosis in tilapia as well as a prophylactic agent in subclinically healthy fish (Najiah et al., 2009). The usage of antibiotics however, can increase the risk of antibiotic resistance to the fish as well as to the human who consume the fish if the withdrawal period is not properly finished.

Among various medicinal plants, *Nigella sativa* (*N. sativa*) (Family Ranunculaceae) is emerging as a miracle herb with a rich historical and religious background since many researches revealed its wide spectrum of pharmacological potential (Aftab Ahmad et al., 2013). The seeds of *N. sativa*, known as black seed, black cumin or "Habatul-Barakah" (Blessed seed), have long used in folk medicine in the Middle and Far East as a traditional medicine for a wide range of illnesses (Schleicher and Saleh, 1998). *N. sativa* has been extensively studied for its biological activities and

therapeutic potential and shown to possess wide spectrum of activities *viz.* as diuretic, antihypertensive, antidiabetic, anticancer and immunomodulatory, analgesic, antimicrobial, anthelmintics, anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, renal protective and antioxidant properties.

1.2 Justification

The study of influence of black seed as antioxidant on oxidative status and the pathology of *Streptococcus agalactiae* infection in red hybrid tilapia was very scarce. Hence, the lack of information on how black seed affects the pathogenesis of experimental *S. agalactiae* infection in red hybrid tilapia induces the need to investigate the post treatment effect of black seed on oxidative stress upon the infection.

1.3 Objectives

i. To evaluate the influence of black seed on the oxidative status of red hybrid tilapia.

ii. To determine severity of histopathological changes of different treatment groups.

1.4 Hypothesis

H_0: Black seed has no ameliorative effects on the oxidative stress and pathology of experimental S. agalactiae infection in red hybrid tilapia.

H_A: Black seed has no ameliorative effects on the oxidative stress and pathology of experimental S. agalactiae infection in red hybrid tilapia.

2.0 LITERATURE REVIEW

2.1 *Nigella sativa*

Nigella sativa is a widely used medicinal plant throughout the world. Al-Dubakel et al. (2012) studied about *N.sativa* as feed additives and proved it had given significant effects on growth and blood glucose of common carp fingerlings. On the hand, the effect of *N. sativa* on immune response of rainbow trout also has been studied by Dorucu et al. (2009). Many researches has been done for human purpose particularly antioxidant effect of *N. sativa*. For example, study done by Mostafa et al. (2013) about antioxidant effect of garlic and black seeds in healthy post-menopausal women revealed significant low levels of plasma MDA with increased erythrocyte glutathione peroxidase and SOD activities.

2.2 Oxidative stress

Oxidation is a natural process that occurs in cellular metabolism in every species. There are mechanisms that are in place to handle the products of oxidation, maintaining oxidative balance within the animal (Dennis et al., 2003). Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. They form as a natural by-product of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis (Cadenas, 1989). Nevertheless, when organisms are exposed to environmental stress (e.g. ultra-violet radiation or heat exposure), ROS can increase extensively causing injury

to cells. When the production and accumulation of ROS is beyond the organism's capacity to deal with these reactive species, there is oxidative stress. This can damage lipids, proteins and deoxyribonucleic acid (DNA) (Halliwell and Gutteridge, 1999). In order to cope with these injuries, cells have antioxidant defences which consist mainly of antioxidant enzymes (AOX), such as superoxide dismutase (SOD), catalase (CAT), glutathione-dependent enzymes (GSH), and non-enzymatic defences such as amino acids, tocopherol and vitamins E, K and C (Martinez-Alvarez et al., 2005; Grim et al., 2010). Malondialdehyde (MDA) is referred as ROS which is also product of lipid peroxidation of polyunsaturated fatty acids (PUFA) in biological samples (e.g. plasma and tissues) (Kinter, 1995; Tsaknis et al., 1999). Furthermore, MDA can be used to assess the extent of lipid peroxidation and widely used biomarker of oxidative stress (Papastergiadis et al., 2012; Rimbach et al., 1999).

2.3 Pathology of *S. agalactiae* infection

The main clinical signs observed in tilapia with streptococcosis by *S. agalactiae* are loss of appetite, unilateral or bilateral exophthalmos, eye haemorrhages, corneal opacity, distended abdomen, curvature of the spinal cord, stiffness, erratic swimming, and bleeding at the base of the fins (Yanong & Francis-Floyd, 2002).

Gross findings in tilapia with streptococcosis are the accumulation of bloody fluid in the abdominal cavity (haemorrhagic ascites), mucous content

with reddish-brown color in the intestine, pale and sometimes enlarged liver, splenomegaly, deposition of a fibrinoid material on the epicardium, and a haemorrhagic brownish appearance of the retro-bulbar tissue and meninges (Pulido et al., 2004; Zamri-Saad et al., 2010).

Microscopically, most tilapias develop a primary inflammatory response of mononuclear cells with the subsequent formation of granulomatous nodules. Lesions include severe meningoencephalitis that can be haemorrhagic and/or granulomatous in nature with large areas of encephalomalacia (softening of the brain tissue); similar tissue lesions are found in the choroid, sclera and the eyeball (Pulido et al., 2004).

The histological sections of liver tissue showed loss of hepatic parenchyma structure, hepatocytes vacoulation and degeneration, hepatopancreas cells degeneration and necrosis and severe blood vessels congestion. Infiltration of eosinophilic granular inflammatory cells around the blood vessels was also observed (Hassan, 2011). Examination of kidney haematopoitec tissue showed cloudy swelling and deposition of hyaline droplets in the tubular epithelial cells and increases presences of haemosiderin deposits (Milud et al., 2013).

3.0 MATERIALS AND METHODS

3.1 Fish and experimental conditions

Healthy cultured red hybrid tilapia weighing about 50 to 150 g were transported from Department of Fishery, Bukit Tinggi, Pahang and reared at the Pathology Laboratory, Universiti Putra Malaysia, Selangor. Prior to the experiment, all tanks were cleaned and disinfected. The water was aerated continuously throughout the study. Hundred and twenty fish were divided into four groups; group A as negative control and B as positive control with total of 40 fish and group C and D, both as treatment groups had 20 fish with duplicates each. The fish approximately of same size were assigned to eight 80 L aquarium. The fish were maintained with 12 h light and 12 h dark per day. Water temperature was checked once a day and they were fed with assigned feed formulation twice a day. The temperature, pH and dissolved oxygen were measured using YSI 556 (YSI, USA). Temperature ranged 28.6 ± 0.1 °C, pH 7.4 ± 0.2, and dissolved 7.02 ± 0.4 mg/L.

3.2 Experimental herb and formulated feed preparation

Black seed was obtained from the local market and added to the commercial feed at 8 % and 15 %. For the formulated feed calculations to balance the nutrient contents, the nutrient requirements of the tilapia, black seed nutrient composition as well as commercial fish feed nutrient composition must be obtained as shown in Table 1, 2 and 3 in Appendices. Then, Pearson square method was being used to balance the formulated diet as described in Appendices. The control diet contained

no supplementation (0 %). The commercial diet and black seed were blended as fine as possible and they were mixed in a mixture machine. The tap water was added intermittently into the mixture by measuring the volume of water needed using the moisture balance. The formulated feeds were pressed through a 2-mm die in a pelleting machine, and the pellets were dried in a drying cabinet (40 °C) until the moisture dropped to around 10 %. It was then stored in vacuum plastic bags and placed in chiller at 3 °C until use.

3.3 Experimental design and feeding trial

Group A and B were fed with 100 % commercial feed or 0 % supplementation of black seed which acted as negative and positive control, respectively. While group C and D were the treatment groups that were fed with 15 % and 8 % of black seed supplementation, accordingly. Feeding trial was carried out about a month before the actual feeding in order to test the degree of palatability of the formulated feed given. The formulations were changed and adjusted until the fish able to eat the feed given. For blood and organs sampling, they were obtained at the day 7 and 14 post-feeding, and post-infection which were at day 17 and day 18 post-feeding. Five fish were sacrificed from each group to collect the organs. The fish were fed with the prepared feed twice a day which were in the morning and late evening.

3.4 Bacterial strains and culture conditions

A bacterium *(S. agalactiae)* was isolated from the brain and kidney of diseased red hybrid tilapia at post mortem examination. Specimens were cultured directly onto horse blood agar at 30 °C for 24 h. The bacterial were Gram-stained and subjected to oxidase and indole tests prior to identification using BBL Crystal ™ Enteric/Non-fermenter ID System (for Gram negative bacteria) and BBL Crystal™ Gram Positive ID System (BBL Crystal, USA). Biochemical test was conducted for the isolate to confirm the identity. Aliquots of *S. agalactiae* were grown on brain heart infusion (BHI) broth (Merck) in a shaking incubator at 37 °C for 18 h. At this time, the cultures were in the stationary phase of growth. Following incubation of the bacterial concentration was determined using the standard plate count technique (Alcamo, 1997). Approximately, 1 mL of the broth was serially diluted ten-fold before 0.1 mL of each dilution was poured and spread onto the blood agar and incubated for 24 h at 30 °C. Following incubation, the number of colonies, particularly those plates containing between 25 to 250 colonies, was counted before the concentration was expressed as colony forming unit (CFU). The final concentration of live *S. agalactiae* was then recorded. For lower concentration, the stock solution 10^7 was then diluted ten-fold using phosphate buffer saline (PBS) and the inocula were immediately taken. The bacterial cells were harvested by centrifugation at 3500 g for 10 min at 4 °C.

3.5 Bacterial challenge and sampling

After 14 days of feeding, five fish per each group (B, C and D) was injected intraperitoneally with 100 µL of *S. agalactiae* of 10^7 CFU/mL for 100 g. Then, the fish were placed into a tank containing fresh water at 28 °C. Blood and organs sampling were performed at day before feeding (D0), a week after feeding (D7), 2 weeks after feeding (D14), 6 hours post-infection (D17), and 18 hours post-infection (D18). At the end of this study, the survival fish were humanely killed and necropsied for bacterial isolation.

3.6 Blood

The collected blood was divided into aliquots. Plasma was harvested after centrifuging the blood at 2054 x g (Centrifuge, 5810R; Eppendorf, Hamburg, Germany). Hemolysates were prepared by wahing the blood with three parts of sterile normal saline and kept at -80 °C until analyzed. The plasma and hemolysates were used for malondialdehyde (MDA) and superoxide dismutase (SOD) analysis, respectively.

3.6.1 Malondialdehyde

The plasma MDA concentration was assayed as per the technique of Ohkawa et al. (1979), with little modifications. Briefly, 2.4 ml of 1/12 h2so4 (Ajax Chemicals, Sydney, NSW, Australia) and 0.3 ml of 10 % sodium tungstate (Na2WO4) (Sigma, St. Louis, MO, USA) were added to 0.3 ml of plasma and centrifuged at 2054 x g

for 10 min. At that point, 0.5 ml of distilled water, 3 ml of 0.05 N HCl (VWR International, Radnor, PA, USA) and 1.0 ml of 1 % thiobarbituric acid (TBA) (MP Biomedicals, Santa Ana, CA, USA) were added and made up to 5 ml with distilled water. The mixture was kept in a water bath at 95 % for 60 min. After cooling, the mixture was centrifuged again at 2054 x g (Centrifuge, 5180R; Eppendorf, Hamburg, Germany), and the supernatant was collected and its absorbency was measured in a spectrophotometer (Optizen 1412v, Banseok-Dong, Yuseong-gu, Daejeon, Korea) at 532 nm. Standard control was made by adding 0.5 ml of distilled water, 3 ml of 0.05 N HCl and 1 ml of 1 % TBA to 0.3 ml of 10 nmol/l 1,1,3,3-tetrahoxypropane (TEP) (Sigma). The absorbency was measured as previously stated. The concentration of MDA was expressed as nmol/l of plasma. The concentration was calculated by mathematical statement:

MDA concentration (nmol/l) = (As/Ab)/ (Vs/Vt)

As = Absorbance of the sample

Ab = Absorbance of the standard control

Vs = Volume of plasma sample

Vt = Volume of the total reactive mixture (ml) in cuvette

3.6.2 Superoxide dismutase

The SOD was measured according to the methods of Marklund and Marklund (1974) as reported by Beutler (1984) with little alteration. Briefly, 500 µL of hemolysate was added to 1.5 mL of ice distilled water accompanied by 0.5mL ethanol (HmbG Germany) and after that 0.3 mL chloroform (R&M Chemicals, Edmonton, UK). The result was blended after every expansion and the last vortex for 30 s and then allowed to stand for 5 min for complete precipitation. The mixture was centrifuged at 2683 x g for 5 min and the supernatant (hemolysate concentration) was collected and kept at -20 °C. At that point 3.9 mL of buffer was added to 70 µL of the hemolysate concentrate, then vortexed and equilibrated with air for 10 min. This was followed by addition of 40 µL of 20 mmol/L pyrogallol solution (Sigma), then mixing and allowed to stand for 30 s. The blank was prepared by replacing the 70 µL of hemolysate with 20 % (v/v) ethanol. Four tubes were prepared and the sample read in 1 min. the enzyme activity was expressed in nmol/mL as:

SOD concentration (nmol/mL) = [{(BΔA-SΔA)/BΔA}/2]/Ve/Vs

BΔA = the rate of spontaneous oxidation of blank control

SΔA = the rate of spontaneous oxidation of sample

Ve = the final volume of extract

Vs = the amount of the sample in the extract

3.7 Organs and histopathological scoring

Samples were examined under light microscope for histopathological scoring particularly examination of the brain and kidneys. The histopathology slide was examined by dividing into 6 different area or fields. Then, the lesions were identified and score accordingly which included 0 = normal, 1 = 30 % of the field affected, 2 = 60 % of field affected, and 3 = more than 60 % of field affected. For each lesion, they were giving the score and total score was accumulated and summarized as percentage in the table form as Table 4. It was then tested at 5 % significance levels using Kruskal-Wallis test and post hoc analysis.

3.8 Polymerase Chain Reaction

Samples of S. agalactiae were obtained from the pure culture colonies from positive bacterial isolation samples. For identification and confirmation of S. agalactiae, total cellular DNA was extracted with Wizard Genomic DNA Purification Kit (Promega, USA) according to manufacturer's protocol. This process was called as DNA extraction. Next, the extracted DNA was evaluated by PCR for S. agalactiae-specific section of 16S-23S Rrna intergenic spacer region with primers ST AUR 4 [ACG GAG TTA CAA AGG ACG AC] and 6 [AGC TCA GCC TTA ACG AGT AC]. Thermal cycler machine was used to perform the PCR cycles which consist of three major steps and repeated for 30 cycles. First step was denaturing at 95 °C for 1 min followed by 95 °C for 15 sec, then second step was annealing at 52 °C for 15 sec and lastly, the third step was extension at 72 °C for 30

sec followed by 72 °C for 2 min. Agarose gel electrophoresis was conducted and the result could be obtained by using the UV transiluminator.

3.9 Statistical analysis

All the statistical analyses were performed using MedCalc for Windows, version 12.7.0.0 (MedCalc Software, Mariakerke, Belgium) and tested at 5 %level of significance. The differences of each time point separately using a one-way analysis of variance (ANOVA) and post-hoc test using the Student-Newman-Keuls pairwise comparison test. The graphs are presented as standard errors of mean (SEM).

4.0 RESULTS

4.1 Mean graph of MDA

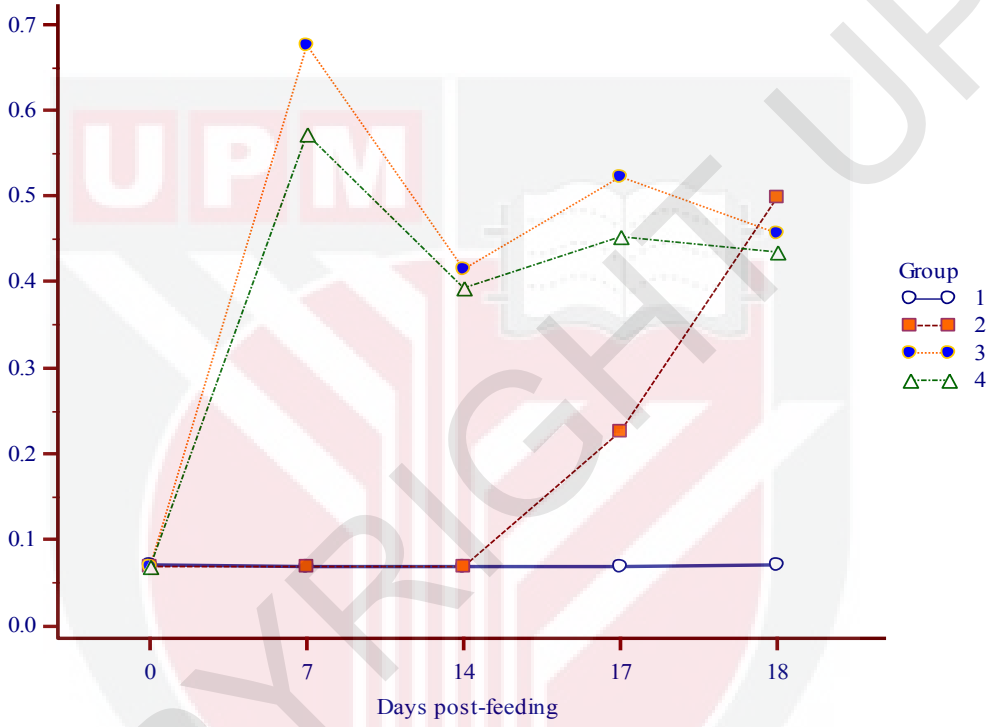


Figure 1. Standard mean value plotted graph with standard error ±0.5 of value of MDA

The mean value of MDA and SOD activities for group A, B, C and D having 0 %, 0%, 15 % and 8 % supplementation of black seed was presented in Figure 1and 2, respectively. The MDA and SOD activities were subjected to analysis of variance revealed significant ($p<0.05$) difference among the groups. Based on the graph of mean value of MDA in Figure 1, there was sudden increase of value for the treatment groups, C and D, compared to the control groups, A and B, at day 7 post-feeding. However, the treatment groups decreased towards day 14 post-feeding and increased gradually at day

17. The graph indicated significant result when the values of MDA of treatment groups were lower compared to the positive control group, B. Group C had higher value of MDA activity if compared with Group D.

4.2 Mean graph of SOD

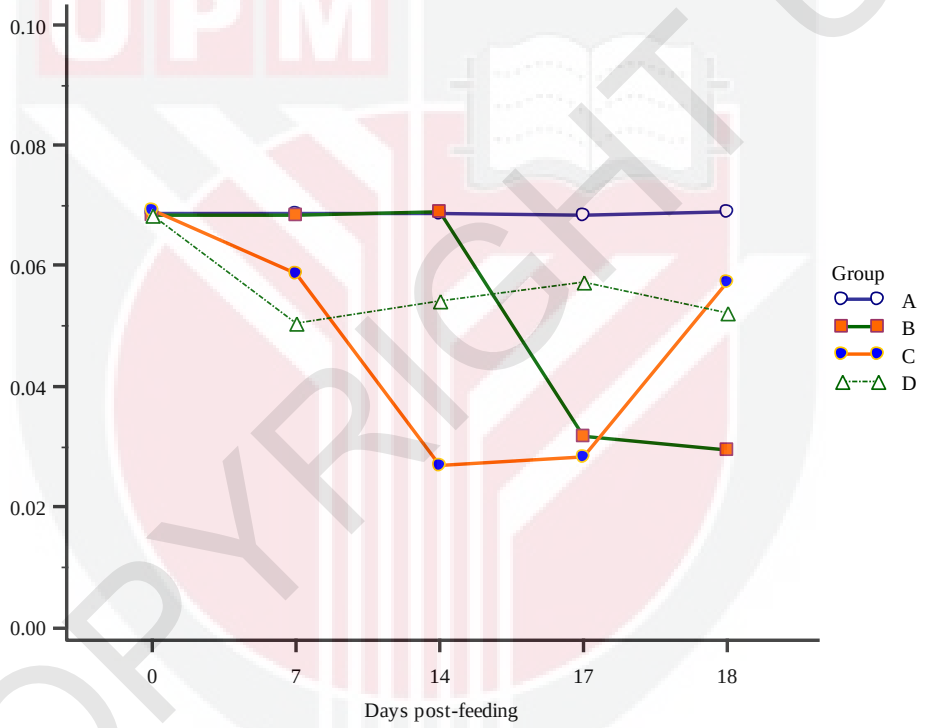


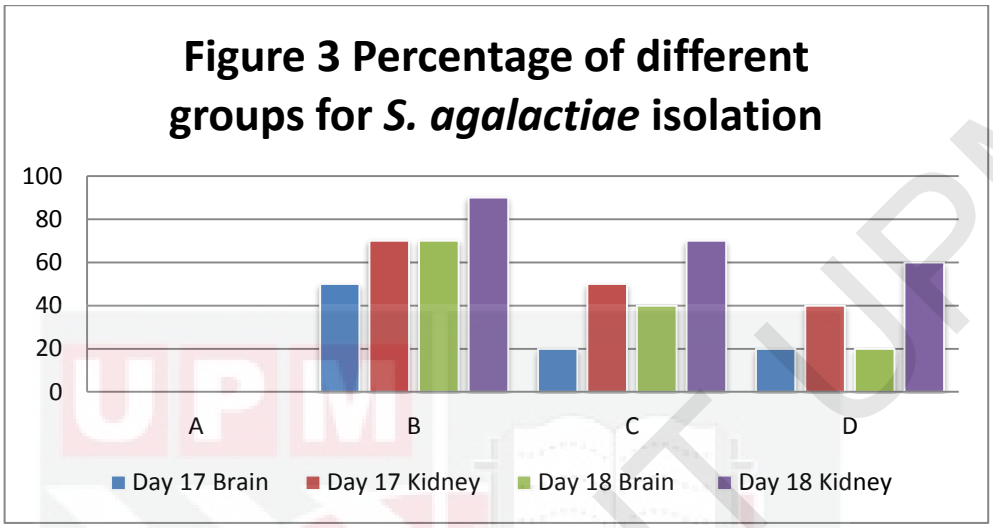
Figure 2. Standard mean value plotted graph with standard error ±0.5 of value of SOD

On the other hand, based on the graph of mean value of SOD as shown in Figure 2 revealed there was marked decrease of value in treatment groups. Group C decrease markedly compare to Group D. The treatment groups also decreasing in value of SOD at day 7 post-feeding. After the challenge, at day 17, the treatment groups increased

compare to Group B. Hence, these produced a significant result. There was no mortality recorded in all groups until the day 18 post-feeding or 18 hours post-infection.

4.3 Microbiology

The sampled organs for bacteriology were brain and kidneys. The primary isolates revealed small pin-point or minute transparent colonies on the blood agar. The colonies were spherical or ovoid in shape and 0.5 to 2.0 µm in diameter. They were characteristically presumptive of Streptococcal spp., occur in pairs or chains, non-motile, non-spore forming and Gram positive. However, only sample from brain and kidneys exposed growth of the colonies and no growth in eye sample. Biochemical profile of the isolates was confirmed by PCR. Figure 3 showed graph of microbiological examination of different groups and organs of red tilapia for *S. agalactiae.* The graph indicated there was bacterial growth for negative control, A and B recorded the highest percentage of growth for both days post-challenge. While C had higher percentage of bacterial growth compared to D. Nevertheless, group B, C and D exposed higher growth at day 17 post-feeding compared to day 18 post-feeding.



4.4 Polymerase Chain Reaction

PCR amplification and sequencing of the s ribosomal ARN gene showed almost all positive result for all the samples taken from the positive bacterial culture colonies as shown in Figure 4.

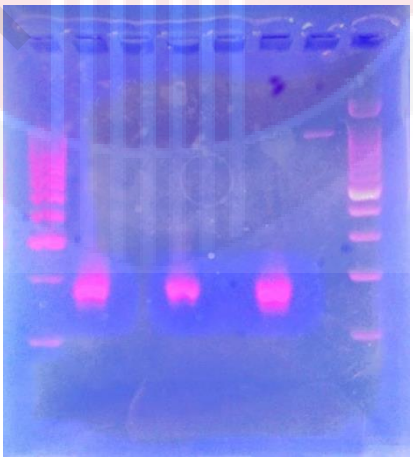


Figure 4. GelRed-stained 2 % agarose gel of multiplex PCR products.

4.5 Histopathology

4.5.1 Histopathological scoring

The scoring was based on the lesions observed from the histopathology examination. The lesions examined in the brain include congestion and inflammatory cells infiltration. Figure 5 indicated the graph of percentage of lesion score for each group for brain with no lesion observed in group A. Group B had the highest lesion score followed by C and D. Group C shown higher percentage of lesion score compared to D.

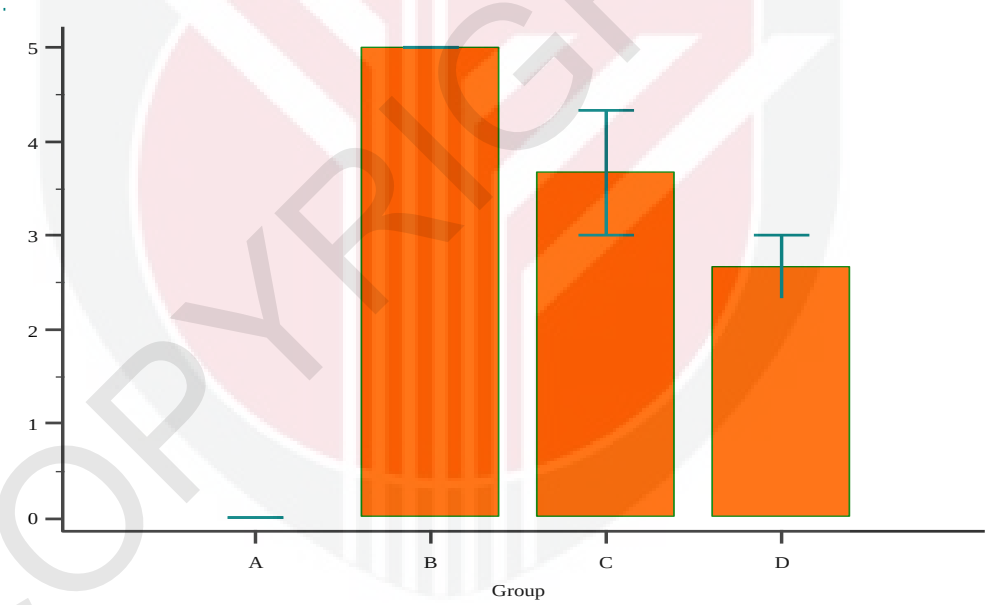


Figure 5. Graph of brain histopathological scoring at Day 18 post-feeding

The representatives from histopathology slides of group C was briefly described in Figure 6 and 7 for brain.

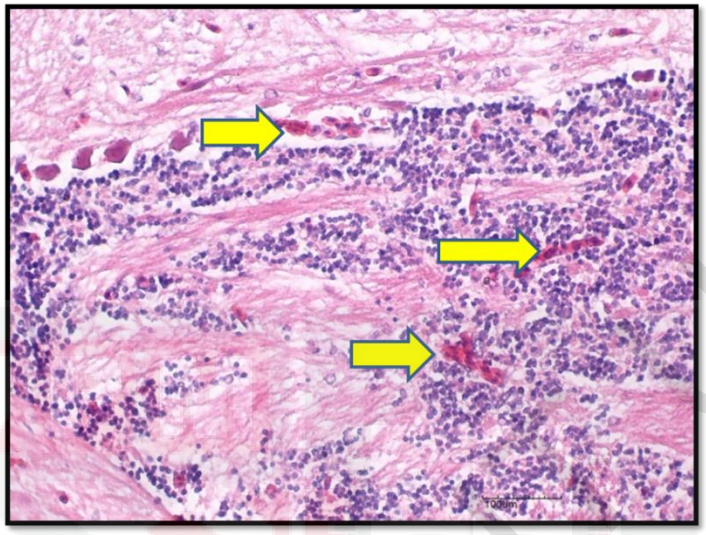


Figure 6. There was congestion (yellow arrows) around the granular cells of the brain.(H&E, x200).

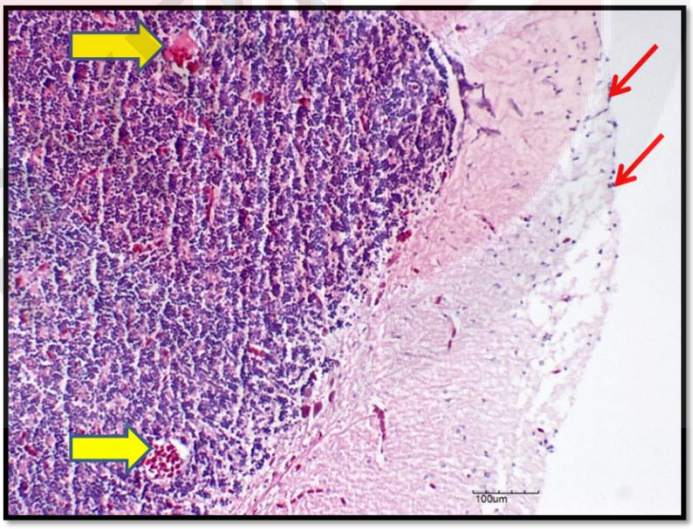


Figure 7. Presence of inflammatory cells at the meninges and congestion at the granular layer (H&E, x200).

Figure 8 indicated the graph of percentage of lesion score for each group with no lesion observed in group A. Group B had the highest lesion score followed by C and D. Group C shown higher percentage of lesion score compared to D. It was more or less the same with the brain histopathological scoring graph. Figure 9 and 10 showed the lesions examined in the kidney include congestion, infiltration of inflammatory cells, tubular necrosis or glomerular atrophy or degeneration.

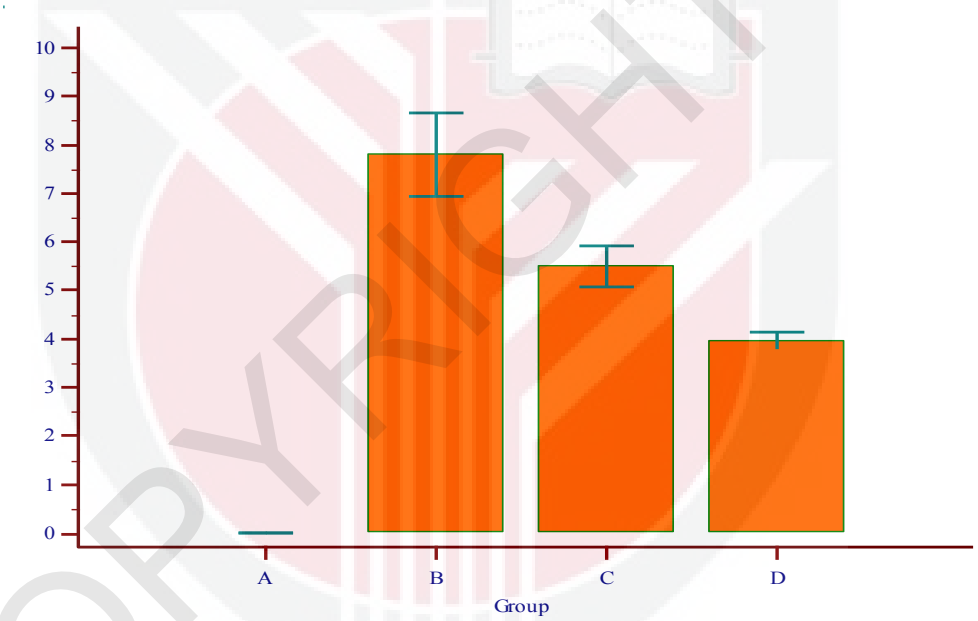


Figure 8. Graph of kidney histopathological scoring at Day 18 post-feeding

The representatives from group B were described histopathologically with some lesions.

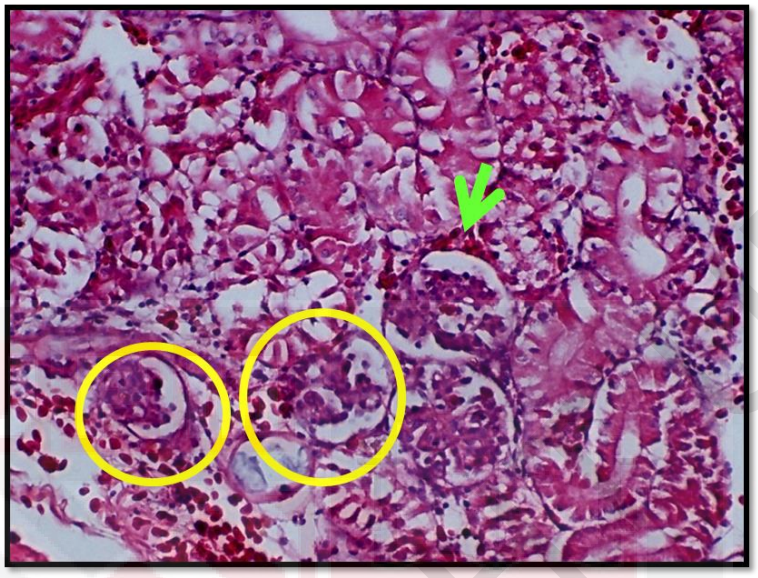


Figure 9. Congestion (green arrow) and presence of glomerular atrophy or degeneration (yellow circle) in kidney. (H&E, x200).

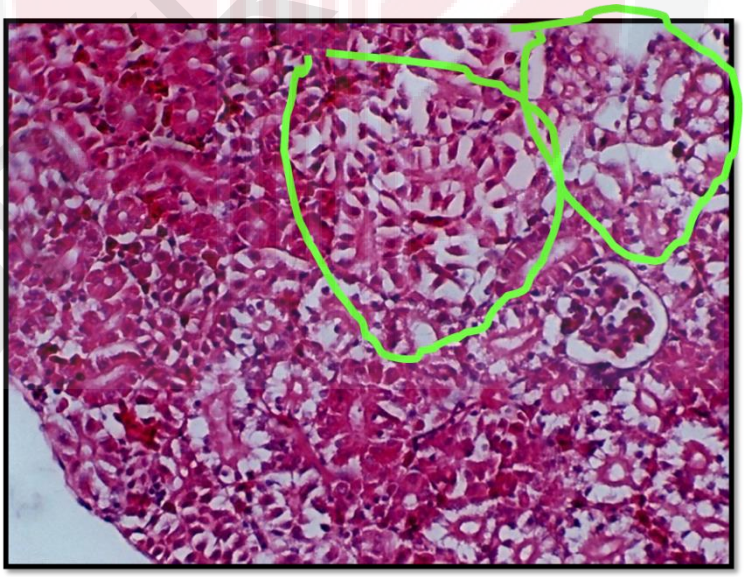


Figure 10. Necrosis of the tubules in kidney tissue. (H&E, x200).

4.6 Statistical Analysis

Table 1 demonstrated the mean value of MDA.

	Mean value of MDA				
	D0	D7	D14	D17	D18
A	0.069	0.068	0.069	0.069	0.069
B	0.069	0.069	0.069	0.225	0.498
C	0.069	0.675	0.416	0.523	0.456
D	0.069	0.573	0.393	0.439	0.436

Table 2 demonstrated the mean value of SOD.

	MEAN VALUE OF SOD				
	D0	D7	D14	D17	D18
A	0.069	0.069	0.069	0.068	0.069
B	0.068	0.068	0.069	0.032	0.029
C	0.069	0.059	0.027	0.028	0.057
D	0.069	0.05	0.054	0.057	0.052

Table 3 depicted brain histopathological scoring at Day 18 post-feeding

Group	Congestion		Infiltration of Inflammatory cells	Total score
A	1	0	0	0
A	2	0	0	0
A	3	0	0	0
B	1	3	2	5
B	2	3	2	5
B	3	3	2	5
C	1	2	1	3
C	2	2	1	3
C	3	3	2	5
D	1	1	2	3
D	2	1	2	3
D	3	1	1	2

Table 4 depicted kidney histopathological scoring at Day 18 post-feeding

Group	Congestion		Infiltration of Inflammatory cells	Tubular necrosis	Glomerular atrophy/degeneration	Total score
A	1	0	0	0	0	0
A	2	0	0	0	0	0
A	3	0	0	0	0	0
B	1	2	1	3	2	8
B	2	1	1.2	1	3	6.2
B	3	2	2	2.2	3	9.2
C	1	1	1.2	1.3	1.4	4.9
C	2	1.13	0.8	1.5	1.8	5.3
C	3	2	1.4	1.2	1.7	6.3
D	1	1	0.8	1	1.3	4.1
D	2	1	0.6	0.6	1.4	3.6
D	3	1	1	0.5	1.7	4.2

5.0 DISCUSSION

Based on the graph of mean value of MDA, sudden increase in the treatment groups, C and D at day 7 post-feeding might be due to the formulated feed that induced stress to the fish. The expected result should be the value of MDA activities will be reduced. The study done by Mostafa et al. (2013) revealed plasma MDA levels showed significant reduction in the plasma of normal postmenopausal women after 8 weeks of garlic and black seeds' consumption. The reason why the value was increased markedly in the study was due the feed was new to the fish and they were not used to the feed. The introduction of feed should be gradual and the period provided to observe the stable changes of MDA activities should be extended more. However, the value decreased gradually towards second week of feeding for treatment groups. This might be due to the fish had been able to adapt to the feed that induced stress. According to Lenartova (1997), fish tend to adapt to oxidative conditions to which they are exposed. Besides, the treatment groups produced significant results at day 17 and 18 post-feeding which was after challenged if compared with group B. This might be due to the protective effect of black seed supplementation in the feed.

Based on the graph of mean value of SOD, marked decrease in value for treatment groups at day 7 post feeding probably due to the normal physiology of the fish where increased of MDA will cause SOD to decrease. Superoxide dismutase is antioxidant enzyme and rapidly converts O_2 to less dangerous H_2O_2 which is further degraded by endogenous antioxidant enzymes GSH-Px and catalase to water (Srividhya

et al., 2008). The feed-induced stress causes SOD to decrease to compensate the MDA activity. However, the levels of SOD for treatment groups after challenged increase significantly compare to group B might be because of the effect of black seed.

No mortality recorded for all groups might be due to low concentration of streptococcal cells used in this study which was 10^7 CFU/mL or the bacteria had loss its virulent properties to cause mortality to the fish.

Bacteriology examination of diseased fish revealed the presence of morphologically and also presence of gram positive, cocci shape in pairs and long chain after gram-stained which were related to *S. agalactiae*. Histopathological scoring revealed that the lesion score at group C was higher than at group D. This might be probably the high amount of black seed do not necessarily show lesser lesion score compare to group D which had less amount of black seed at day 18 post-feeding. This statement applied to both brain and kidney.

The summary for histopathological findings particularly for brain was congestion at the granular layer of the brain and also infiltration of the inflammatory cells at the meninges. Abuseliana et al. (2011), brain blood vessels were congested and some exhibit dilation and detachment of blood vessels wall. Meningitis and thickening of meninges were also observed in most brain samples. In addition, the lesions found in the kidney were congestion, infiltration of the inflammatory cells, tubular degeneration as well as glomerular atrophy or degeneration. Kidney hematopoietic tissue showed various degree of degeneration (Abuseliana et al., 2011).

6.0 CONCLUSION AND RECOMMENDATIONS

The result of this experiment showed that the oxidative stress level of treatment groups, C and D, able to produce significant result. The protective effect after challenge showed incorporation of black seed in fish feed could be seen in this study. However, group D that had lower amount of black seed (8 %) able to provide better result the MDA and SOD activity levels showed stable and optimal effect if compared to group C that had higher amount of black seed (15 %) ae well as the result in histopathological scoring. Hence, incorporation of black seed in fish feed could ameliorate the disease condition in the fish particularly with *S. agalactiae* infection.

This project is recommended for future study since there are many of black seed properties or effects that can be learned particularly in aquaculture industry. For this project, some suggestions are to improve the palatability of the formulated feed. Besides, it is recommended to extend the feeding period to at least 21 days since many literature reviews perform the minimum feeding trial period is 21 days. The period to perform this project also should be extended to examine the histopathological changes in other organs such as eyes, liver and spleen. Finally, in order to evaluate the mortality rate of the fish in the future, the concentration of streptococcal cells used to infect should be increased or the bacteria can be passaged until the infected fish shows signs of streptococcosis.

REFERENCES

A.M. Adly, A. (2010). Oxidative Stress and Disease: An Updated Review. *Research J. Of Immunology*, *3*(2), 129-145. doi:10.3923/rji.2010.129.145

Abdullah, S., Omar, N., Yusoff, S., Obukwho, E., Nwunuji, T., Hanan, L., & Samad, J. (2013). Clinicopathological features and immunohistochemical detection of antigens in acute experimental Streptococcus agalactiae infection in red tilapia (Oreochromis spp.). *Springerplus*, *2*(1), 286. doi:10.1186/2193-1801-2-286

Abuseliana, A., Mohd Daud, H., Abdul Aziz, S., Bejo, S., & Alsaid, M. (2011). Pathogenicity of Streptococcus agalactiae Isolated from a Fish Farm in Selangor to Juvenile Red Tilapia (Oreochromis sp.). *J. Of Animal And Veterinary Advances*, *10*(7), 914-919. doi:10.3923/javaa.2011.914.919

El-Sayed, A. (2006). *Tilapia culture*. Wallingford, UK: CABI Pub.

Fao.org,. (2015). *FAO Fisheries & AquacultureÂ Oreochromis niloticus*. Retrieved 8 March 2015, from http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en

Mostafa, R., Moustafa, Y., Mirghani, Z., AlKusayer, G., & Moustafa, K. (2013). Antioxidant effect of garlic (Allium sativum) and black seeds (Nigella sativa) in healthy postmenopausal women. *SAGE Open Medicine*, *1*(0). doi:10.1177/2050312113517501

Musa, N., Wei, L., Musa, N., Hamdan, R., Leong, L., & Wee, W. et al. (2009).

Streptococcosis in red hybrid tilapia (Oreochromis niloticus) commercial farms in Malaysia. *Aquaculture Research, 40*(5), 630-632. doi:10.1111/j.1365-2109.2008.02142.x

Najiah, M., Aqilah, N., Lee, K., Khairulbar, Z., Mithun, S., & Jalal, K. et al. (2012). Massive Mortality Associated with Streptococcus agalactiae Infection in Cage-cultured Red Hybrid Tilapia Oreochromis niloticus in Como River, Kenyir Lake, Malaysia. *J. Of Biological Sciences, 12*(8), 438-442. doi:10.3923/jbs.2012.438.442

Pathologicl Findings of Experimental Streptococcus Agalactiae Infection in Red Hyprid Tilapia (Oreochromis sp.). (2014). *International Journal Of Advances In Chemical Engineering And Biological Sciences*, *1*(1). doi:10.15242/ijacebs.c1213075

Yang, W., & Li, A. (2009). Isolation and characterization of Streptococcus dysgalactiae from diseased Acipenser schrenckii. *Aquaculture, 294*(1-2), 14-17. doi:10.1016/j.aquaculture.2009.05.018

Zamri-Saad, M., Amal, M., & Siti-Zahrah, A. (2010). Pathological Changes in Red Tilapias (Oreochromis spp.) Naturally Infected by Streptococcus agalactiae. *Journal Of Comparative Pathology*, *143*(2-3), 227-229. doi:10.1016/j.jcpa.2010.01.020

Al-Dubakel, A. Y., Al-Mhawe, B. H., Shaeyal, L. W., & Majeed, M. F. (2012). Preliminary study on the effect of dietary black seed (Nigella sativa) on growth and blood glucose of common carp (Cyprinus carpio) fingerlings. *J .of Thi _Qar*

Univ. for Agri.Researches, 1(2), 42. Retrieved from http://www.iasj.net/iasj?func=fulltext&aId=56688

Nwunuji, T., Mayowa, O., Yusoff, S., Bejo, S., Salisi, S., & Mohd, E. (2014). The ameliorative effect of ascorbic acid on the oxidative status, live weight and recovery rate in road transport stressed goats in a hot humid tropical environment. *Animal Science Journal, 85*(5), 611-616. doi:10.1111/asj.12174

Iregui, C., Barato, P., Rey, A., Vasquez, G., & Verjan, N. Epidemiology of Streptococcus agalactiae and Streptococcosis in Tilapia Fish.

Dorucu, M., Colak, S. O., Ispir, U., Altinterim, B., & Celayir, Y. (2009). The effect of black cumin seeds, Nigella sativa, on the immune response of rainbow trout, Oncorhynchus mykiss. *Med. Aquacult. J, 2*(1), 1-7.

Marklund SL, Marklund G. 1974. Superoxide dismutase assay by auto-oxidation of Pyrogallol. *European Journal of Biochemistry* **47,** 469–474.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical of Biochemistry 95, 351–358.

Siti-Zahrah, A., Misri, S., Padilah, B., Zulkafli, R., Kua, B. C., Azila, A. And Rimatulhusna, R. (2004). Predisposing factors associated with outbreak of Streptococcal infection in floating cage-cultured red tilapia in reservoirs. Pp. 129.

APPENDICES

Source: Tilapia Environmental Biology and Nutritional Requirements (South Dakota Cooperative Extension Service)

TABLE 1: NUTRIENT REQUIREMENTS OF TILAPIA (*Oreochromis niloticus*/Nile tilapia)

Protein (dry basis)	28-30 %
Dietary lipid	4-8 %
Carbohydrate	30-40 %
Crude fibres	8-10 %
Protein to energy ratio (mg/Kcal)	68-125

TABLE 2: PROXIMATE COMPOSITION OF BLACK CUMIN SEEDS

Proximate composition (%)	
Moisture	6.46 ± 0.17
Crude protein	22.8 ± 0.6
Crude fat	31.16 ± 0.82
Crude fibre	6.03 ± 0.16
Ash	4.20 ± 0.11
Nitrogen Free Extract	29.36 ± 0.78

(Muhammad Tauseef Sultan et al., 2009)

TABLE 3: PROXIMATE COMPOSITION OF COMMERCIAL OR PELLET FEED FOR FINISHER

	LABEL FROM FISH SHOP	FAO WEBSITE
Crude protein	Min 18%	20%
Moisture	Max 10%	-
Crude fat	Min 5%	4%
Crude fibre	Max 6%	5.2%

PEARSON SQUARE METHOD

Ingredients **Proportion**		**On % basis**	**On a 1000 g Basis**
Commercial feed			
34	**7**	(7/11) x 100 = 63.6 %	63.6/100 x 1000 g = 636 g
	30		
23	**4**		
Black seed parts	**= 11**	(4/11) x 100 = 36.4 %	36.4/100 x 1000 g = 364 g

Thus, mixing 63.6 % of commercial feed (34 % CP) and 36.4 % of black seed (23 % CP) will provide a mix of 30 % CP.

Check: to ensure that desired CP is met

Commercial feed = 34/100 x 63.6 = 22

Black seed = 23/100 x 36.4 = 8

→ Total CP in mix: 22 + 8 = 30 % → mix balanced for CP content

1. **First feed formulation for feed trial using crude protein (CP)**

Commercial feed	Black seed
636 g	364 g
818 g	182 g

2. **Second trial using different formulations → USE FOR ACTUAL FORMULATED FEED FORMULATION**

Commercial feed	Black seed
850 g	150 g
925 g	75 g